Remarks

Claims 119-143 are pending. Claims 119, 126-127, 129, 131 and 132 are amended. The amendments are supported by the application as filed, thus there is no issue of new matter. Claims 120-125, 128 and 143 are cancelled without disclaimer or prejudice to applicants' right to pursue patent protection for the subject matter thereof in another application. Claims 119, 126-127 and 129-142 thus appear in the application. Entry of this Amendment is respectfully requested as it is believed to place the application in condition for allowance, or at a minimum to materially reduce the issues for an appeal.

Support for the claim amendments is found, *inter alia*, in the specification as follows: **Claim 119**: page 12, lines 15-16, page 13, lines 8-10, page 14, lines 1-5 and page 53, line 35 to page 54, line 4; **Claims 126-127**: dependencies changed from claim 125 to claim 119 due to the cancellation of claim 125; **Claim 129**: page 13, lines 8-10; **Claim 131**: page 12, lines 15-16, page 13, lines 8-10, page 14, lines 1-5 and page 53, line 35 to page 54, line 4; and **Claim 132**: page 12, lines 15-16, page 13, lines 8-10, page 14, lines 1-5, page 15, line 27 to page 16, line 5, page 53, line 35 to page 54, line 4, and the Experimental Results discussed at pages 36-118.

Applicants note their appreciation for the courtesies extended by Examiner Holleran and her Supervisor, Examiner Anthony Caputa, to their representatives, John P. White, Esq. and Mark A. Farley, Esq. during a telephone interview concerning this application on Tuesday, December 2, 2003. The amendments and comments submitted in this Response are in accordance with the matters discussed during that telephone interview and thus constitute a written record thereof.

REJECTIONS WITHDRAWN

In ¶4 of the Office Action the Examiner stated that the provisional double-patenting rejection of claims 119-143 over application No. 08/475,084 is withdrawn because the claims of the instant application are drawn to conjugates comprising a GM2 ganglioside, whereas those of 08/475,084 are drawn to conjugates comprising a GD3 ganglioside.

In ¶5 of the Office Action the Examiner stated that the provisional double-patenting rejection of claims 119-143 over application No. 08/477,147 is withdrawn because the claims of the instant application are drawn to conjugates comprising a GM2 ganglioside, whereas those of 08/477,147 are drawn to conjugates comprising a GD2, GD3 lactone, O-acetyl GD3 or GT3 ganglioside.

OBJECTIONS/REJECTIONS MAINTAINED

The Examiner stated in ¶6 of the Office Action that the prior objection to the disclosure is maintained for the reasons set forth in the Office Action mailed 6/19/98 (Paper No. 16). The Examiner stated that Applicants submit they will provide a new Figure 6B to overcome the rejection when the case is in condition for allowance, but until applicants submit a proper Figure, the objection is maintained.

In Paper No. 16, the Examiner stated that on page 5, line 30 of the application, in the Brief Description of the Figures, Figure 6b is listed as graphing IgG antibodies but Figure 6b has the Y-axis labeled as IgM titer. The Examiner stated that the appropriate correction is required.

Attached hereto as **Exhibit A** is an annotated (marked-up) copy of Figure 6B indicating in red ink a proposed change wherein the Y-axis

is now labeled as "IgG". **Exhibit B** is a replacement drawing sheet with the above-indicated change made to the labeling of the Y-axis. The amendment to Figure 6B is supported by page 5 of applicants' specification. Applicants respectfully request entry of the drawing correction as it raises no issue of new matter. The Examiner is requested to reconsider and withdraw the objection to the disclosure in view of the submission of the corrected Figure.

Double-Patenting Rejection

In ¶7 on page 3 of the Office Action the Examiner stated that the provisional rejection of claims 119-143 for obviousness-type double patenting over claims 101-126 of Application No. 08/477,097 is maintained for the reasons of record, as applicants argue only that the rejection should be withdrawn if the claims are found allowable.

In response to this provisional rejection, submitted herewith as **Exhibit C** is a Terminal Disclaimer over any patent issued from U.S. Serial No. 08/477,097. The disclaimer has been executed by an authorized representative of Sloan-Kettering Institute for Cancer Research, i.e., the Assignee of both the subject application and U.S. Serial No. 08/477,097. As set forth in §804 IB of the Manual of Patent Examining Procedure, the "provisional" double-patenting rejection will become an "actual" double-patenting rejection if U.S. Serial No. 08/477,097 issues as a patent prior to or on the same date as the subject application. The effect of the terminal disclaimer would be to prevent the term of any patent issuing based on the subject application from extending beyond the term of the patent based on U.S. Serial No. 08/477,097. The Examiner is respectfully requested to reconsider and withdraw the provisional double-patenting rejection of claims 119-143.

Rejections Under 35 U.S.C. §103(a)

In ¶8 of the Office Action claims 119-129 are rejected under 35 U.S.C. 103(a) over the combination of six references, i.e., Wiegand et al (U.S. Patent 5,599,914) in view of Fiume et al (Critical Rev. Therapeutic Drug Carrier Systems, 4(4):265-284, 1988), Ritter et al. (Seminars in Cancer Biology, 2:401-409, 1991), Kensil et al. (The Journal of Immunobiology, 146(2):431-437, 1991), Marciani et al. (Vaccine, 9:89-96,1991) and Uemura et al. (J. Biochem, 79(6):1253-1261, 1976).

In ¶9 claims 119, 129-132 and 134-143 are rejected under 35 U.S.C. 103(a) over the combined disclosure of eight references, i.e., Wiegand et al (U.S. Patent 5,599,914), Fiume et al (Critical Rev. Therapeutic Drug Carrier Systems, 4(4):265-284, 1988), Livingston et al. (Cancer Research, 149:7045-7050, 1989) in view of Ritter et al. (Seminars in Cancer Biology, 2:401-409, 1991), Livingston et al. (U.S. Patent No. 5,102,663), Kensil et al. (The Journal of Immunology, 146(2):431-437, 1991), Marciani et al. (Vaccine, 9:89-96, 1991) and Uemura et al. (J. Biochem, 79(6):1253-1261, 1976).

In ¶10 claims 132 and 133 are rejected under 35 U.S.C. 103(a) over the eight references discussed in the paragraph above in combination with Irie et al. (U.S. Patent No. 4,557,931).

It is respectfully noted that the nine references relied upon, in combination, to reject the claims under 35 U.S.C. §103(a) have been discussed in detail in, *inter alia*, applicants' Amendment in Response to December 31, 2002 Office Action filed April 4, 2003, wherein applicants described several features which they submit distinguish the invention. Those arguments are incorporated by reference herein and thus are not repeated here. Applicants have now, moreover, amended the claims such that, as discussed below they

now recite several additional features which patentably distinguish the invention over the prior art.

The Inclusion Of The Adjuvant QS-21 Provides Unexpected Results That Demonstrate The Non-Obviousness Of The Invention

Composition claims 119, 129 and method claims 131-132 were amended to specifically recite that the composition includes the adjuvant QS-21, i.e., a saponin derivable from the bark of a Quillaja saponaria Molina tree. During the December 2, 2003 interview, Applicants' representative directed the Examiner's attention to pages 94-95 of the application, which describe unexpected results achieved with compositions as claimed including QS-21 as an adjuvant in comparison to those obtained with corresponding compositions containing prior art adjuvants, i.e., BCG and DETOX.

Briefly, the specification teaches that local reactions to dosages of 100-200 μg of QS-21 were "quite different" (p.94, line 5) than those seen with comparable dosages of BCG and DETOX. It further states that the local response is more diffuse than the response generally seen with doses of DETOX or BCG inducing comparable systemic symptoms (lines 8-11). It additionally teaches (lines 11-16) that a surprising feature of the subjects' response to QS-21 was that several days later (at most 10 days later) the local reactions had completely abated and there was no evidence that the vaccination had been administered to that site.

Applicants' specification additionally teaches (see paragraph bridging pps. 94-95) that QS-21, at any of the dosages used, resulted in a qualitatively different response than those achieved with the prior art adjuvants to GM2 ganglioside. The results obtained with QS-21 were contrasted with the immunogenic response achieved with the use of GM2-KLH vaccines alone or with optimal

doses of BCG or DETOX, which were demonstrated to be substantially less effective than comparable compositions including QS-21. The specification additionally teaches that the results achieved by applicants demonstrate that the 100 and 200 μg doses of QS-21 induce the optimal antibody response against GM2 and that the 100 μg dose is better tolerated. These dosages are specifically recited in applicants' claims.

To summarize, applicants contend that the inclusion of QS-21 in their claimed compositions produces two unexpected improvements over the results achieved with the prior art BCG and DETOX adjuvants: (1) the side effects attributed to such adjuvants are ameliorated with the use of QS-21; and (2) even at the lowest doses of the QS-21 adjuvant, all of patients tested produced IgG antibodies against GM2. Applicants' independent claims, as amended, specifically recite the presence of the QS-21 adjuvant in an amount between about 10 μg and about 200 μg. Applicants contend that these recitations patentably distinguish the invention from the prior art.

During the December 2, 2003 interview the Examiner inquired whether the above-described results attributable to the inclusion of QS-21 were truly unexpected in light of the disclosure of the Kensil et al. reference. Applicants' representative pointed out that the reference does not suggest the use of QS-21 as an adjuvant and, in fact, teaches away from such use. For instance, page 435 of Kensil et al. makes clear that QS-7 adjuvant is more advantageous than QS-21 in that QS-7 is both less toxic and less hemolytic than QS-21. These advantages of QS-7 over QS-21 are important since the adjuvant is to be combined with the conjugate (discussed below) for administration to human subjects to stimulate or enhance the production of antibodies and/or to treat a human subject having cancer. Clearly, the increased toxicity and hemolytic activity of

QS-21 disclosed in Kensil et al. teaches away from the use of QS-21 and toward the use of QS-7. In summary, Kensil et al. would not lead one of ordinary skill in this art to expect the surprising results achieved using QS-21 as the adjuvant, which results demonstrate the non-obviousness of applicants' claimed invention.

The Claimed Conjugate And The Molar Ratio Of Conjugated Ganglioside Derivative To Keyhole Limpet Hemocyanin Provide Additional Evidence Of Patentability

The claims recite, *inter alia,* (1) a conjugate of a GM2 ganglioside derivative and Keyhole Limpet Hemocyanin, and (2) a GM2:Keyhole Limpet Hemocyanin molar ratio from 200:1 to 1400:1. These features further patentably distinguish the invention from the prior art cited to reject the claims.

The primary reference cited by the Examiner is U.S. Patent No. 5,599,914 to Wiegand et al. ("Wiegand"). Wiegand discloses at col. 7, lines 1-8 that ganglioside derivatives (GM3, GD3, GM2 and GM1) were reacted with Human Serum Albumin, i.e., not Keyhole Limpet Hemocyanin as recited in applicants' claims, and that the HSA was derivatized with 16-18 SPDP molecules. The reference also teaches that this level was the preferred level (see, e.g., col. 7, lines 1-3). In contrast, applicants' claims recite a GM2:Keyhole Limpet Hemocyanin molar ratio of between 200:1 and 1400:1. Such a ratio is neither taught nor suggested by Wiegand. The subject reference teaches away from the invention due to its teaching that the derivatization level of 16-18 is the "desired" level, and in view of the use of Human Serum Albumin as the carrier instead of Keyhole Limpet Hemocyanin as recited in applicants' claims. There is no disclosure in the reference, moreover, which would suggest the replacement of Human Serum Albumin with Keyhole Limpet Hemocyanin, or to produce a conjugate having a derivatization level different than that disclosed in Wiegand.

The Examiner combined Wiegand with Fiume et al. ("Fiume"), stating that "Wiegand in combination with Fiume teaches a glycoconjugate as claimed in claims 119 or 129." Applicants respectfully traverse this contention. The portion of Fiume cited by the Examiner (commencing at page 268) states that a **drawback** to the clinical use of the conjugates disclosed therein is their immunogenicity. The thrust is therefore to find a methodology for reducing the immunological effect of these conjugates. This teaching is opposite to that provided by the applicants about their invention in that the purpose of the conjugates of the present invention is to increase, **not** to reduce, the immunogenic effect (see, e.g., claim 131).

Fiume not only teaches away from the present invention, it contains no disclosure which would suggest its combination with Wiegand. Wiegand discloses the formation of a composition for use in producing an immunogenic response. In contrast, Fiume teaches to proceed in a diametrically opposed direction, i.e., to seek compounds having a reduced immunogenic effect. These contrasting teachings would lead a skilled artisan away from combining Wiegand with Fiume. Further, even if combined, such combination would not produce the claimed glycoconjugate.

The Improved Results Obtained With Applicants' Compositions Evidence The Patentability Of These Compositions

Applicants provided a reference by Chapman et al., Clinical Cancer Research, Vol. 6, pp. 874-879 (March 2000) entitled, "Induction of Antibodies Against GM2 Ganglioside By Immunizing Melanoma Patients Using GM2-Keyhole Limpet Hemocyanin + QS21 Vaccine: A Dose-Response Study" (hereinafter "Chapman") as Exhibit E to their Amendment filed April 12, 2002. Another copy of this reference is provided as **Exhibit D** hereto.

As noted at page 24 of applicants' April 12, 2002 response, in clinical trials melanoma patients vaccinated with GM2-KLH + QS-21 (i.e., the claimed composition) made using the conjugation procedure described in the present application, produced high titer IgM and IgG antibodies specific for GM2. These clinical results led the authors (including Dr. Philip O. Livingston, a co-inventor of the present invention) to state that the GM2-KLH/QS-21 composition, formulated as presently claimed, "is more immunogenic than our previous formulation." (see Abstract). The "previous formulation" comprised GM2 and *bacilli Calmette-Guerin* (BCG).

The Livingston paper and the Livingston '663 U.S. Patent both disclose the GM2-BCG formulation, i.e., the "previous formulation". The improved formulation described in the present application and claimed herein is distinguishable thereover in that the "previous formulation" does not comprise the same components as the compositions recited in applicants' claims. Further, not only are applicants' formulations made with different components, the present claims additionally recite specific ranges for the components included in these formulations. As demonstrated in Chapman, the presently claimed compositions produce significantly improved results in contrast to those achieved with the previous formulations.

In summary, the claimed compositions are distinguishable over those in the Livingston references as, due to (1) differences in the components from which they are formed, and (2) the relative amounts of the conjugate and the saponin in the claimed compositions, as taught by Chapman they produce a substantially improved immune response to subjects in a clinical setting.

The remaining references cited by the Examiner, i.e., Ritter et al., Marciani et al., Uemura et al. and Irie et al., do not remedy the

deficiencies of the references discussed above. Applicants submit that the claimed invention is patentably distinguishable over all of the cited references and respectfully request the Examiner to reconsider and withdraw the rejections of the claims under 35 U.S.C. §103(a).

NEW GROUNDS OF REJECTION

In ¶12 of the Office Action claims 122-124 and 143 are rejected under 35 U.S.C. §112, first paragraph, as failing to comply with the written description requirement.

The Examiner stated that claims 122-124 recite ranges that are not described in the specification. Claims 122-124 are cancelled herein and thus the §112 rejection of those claims is moot.

The Examiner stated that claim 143 is drawn to a method for delaying recurrence of melanoma, and that the passages pointed to by applicant as providing support do not teach the recited references and do not teach methods for delaying the recurrence of melanoma. In response, claim 143 has been cancelled and thus the rejection of that claim is also moot.

SUMMARY

For the reasons set forth, applicants respectfully request that the Examiner reconsider and withdraw the various grounds of objection and rejection and earnestly solicit allowance of the now pending claims.

If a telephone interview would be of assistance in advancing prosecution of the subject application, applicants' attorneys invite the Examiner to telephone either of them at the number provided below.

A check for FIVE HUNDRED AND THIRTY DOLLARS ($530.00) is enclosed herewith. This amount has been determined by adding the fee of $475.00 due for the three-month extension of time to the fee of $55.00 due for filing the Terminal Disclaimer ($475.00 + $55.00 = $530.00). If any additional fees are required, authorization is hereby given to charge the amount of such required fee(s) to Deposit Account No. 03-3125.

Respectfully submitted,

John P. White
Registration No. 28,678
Mark A. Farley
Registration No. 33,170
Attorneys for Applicant(s)
Cooper & Dunham, LLP
1185 Avenue of the Americas
New York, New York 10036
(212) 278-0400

I hereby certify that this correspondence is being deposited this date with the U.S. Postal Service with sufficient postage as first class mail in an envelope addressed to: Mail Stop AF, Commissioner for Patents, P.O. Box 1450, Alexandria, VA , 22313-1450

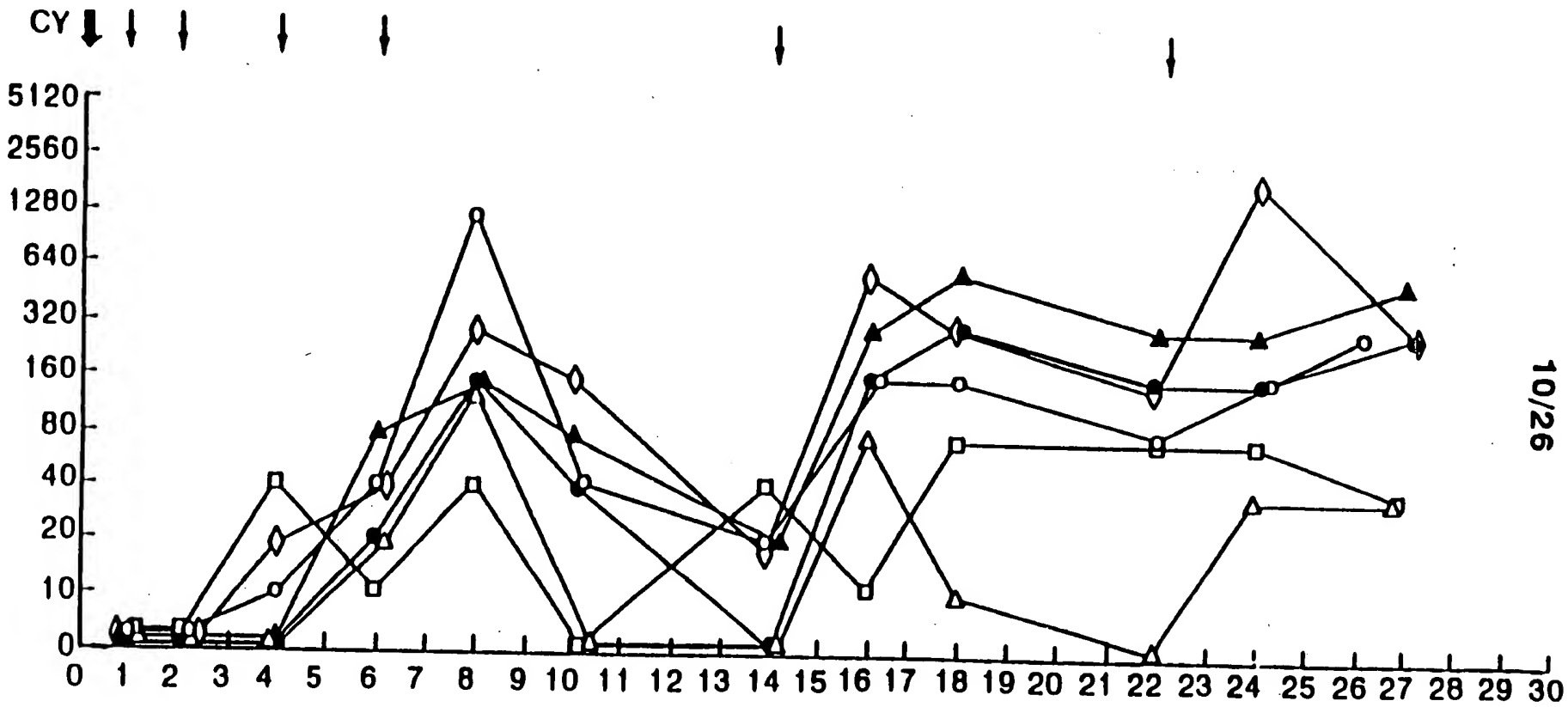
12-10-05

John P. White Date
Reg. No. 28,678
Mark A. Farley
Reg. No. 33,170

RECIPROCAL ~~IgM~~ IgG TITER AGAINST GM2 by ELISA

GM2-KLH plus QS-21 (70 mcg GM2)

FIGURE 6B



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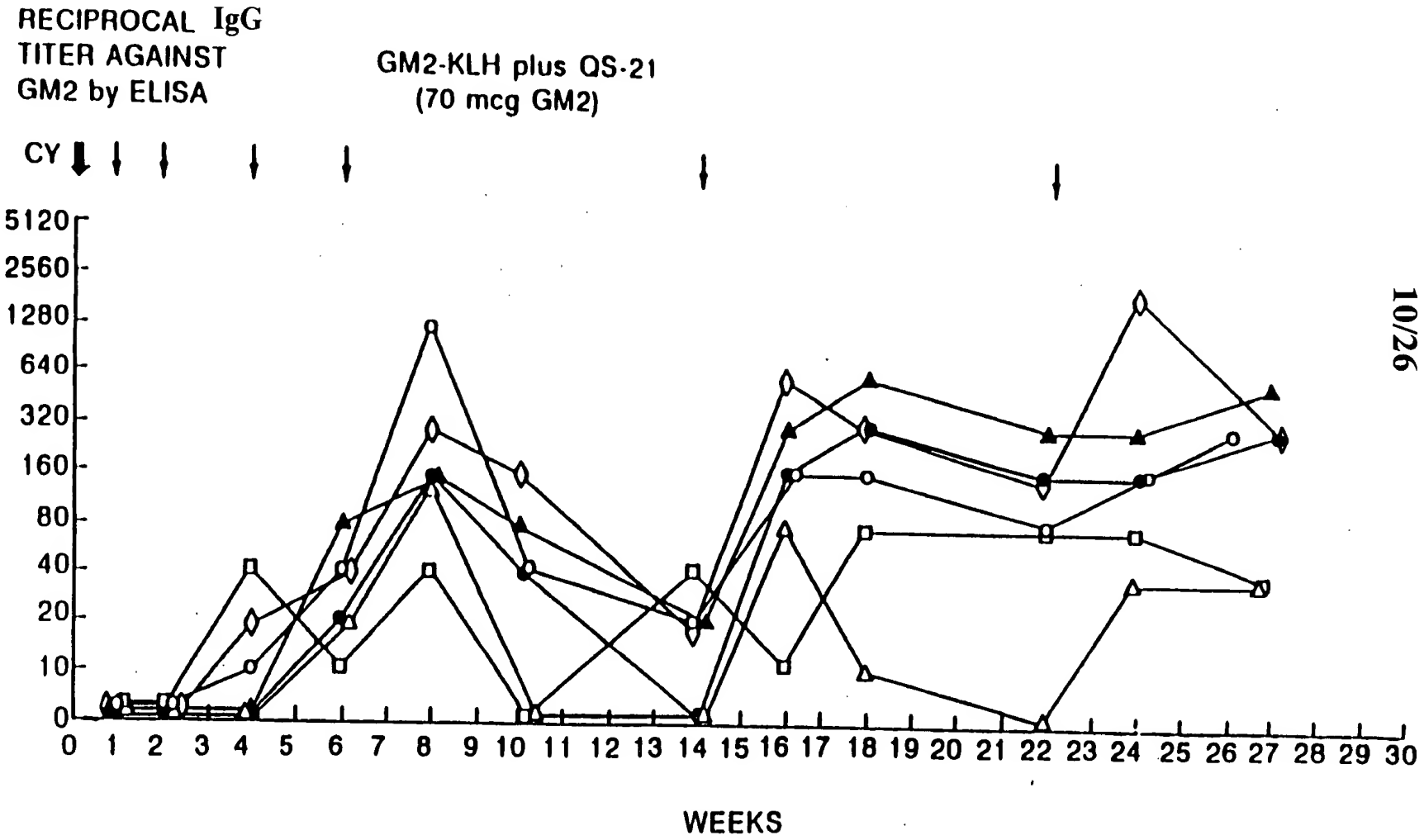
"Annotated Marked-up Drawings"

FIGURE 6B



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"Replacement Sheet"

Induction of Antibodies against GM2 Ganglioside by Immunizing Melanoma Patients Using GM2-Keyhole Limpet Hemocyanin + QS21 Vaccine: A Dose-Response Study[1]

Paul B. Chapman,[2] D. M. Morrissey, K. S. Panageas, W. B. Hamilton, C. Zhan, A. N. Destro, L. Williams, R. J. Israel, and P. O. Livingston[3]

Department of Medicine, Clinical Immunology Service, [P. B. C., A. N. D., L. W., P. O. L.], and Department of Epidemiology and Biostatistics [K. S. P.], Memorial Sloan-Kettering Cancer Center, New York, New York 10021, and Progenics Pharmaceuticals, Inc., Tarrytown, New York [D. M. M., W. B.H., C. Z., R. J. I.]

ABSTRACT

In a previous randomized Phase III trial (P. O. Livingston *et al.*, J. Clin. Oncol., *12:* 1036–1044, 1994), we demonstrated that immunization with GM2 and *bacille Calmette-Guérin* reduced the risk of relapse in stage III melanoma patients who were free of disease after surgical resection and who had no preexisting anti-GM2 antibodies. That vaccine formulation induced IgM anti-GM2 antibodies in 74% but induced IgG anti-GM2 antibodies in only 10% of the patients. To optimize the immune response against GM2, a reformulated vaccine was produced conjugating GM2 to keyhole limpet hemocyanin (KLH) and using the adjuvant QS21 (GM2-KLH/QS21). In pilot studies, 70 μg of vaccine induced IgG anti-GM2 antibodies in 76% of the patients. We wished to define the lowest vaccine dose that induced consistent, high-titer IgM and IgG antibodies against GM2. Fifty-two melanoma patients who were free of disease after resection but at high risk for relapse were immunized with GM2-KLH/QS21 vaccine at GM2 doses of 1, 3, 10, 30, or 70 μg on weeks 1, 2, 3, 4, 12, 24, and 36. Serum collected at frequent and defined intervals was tested for anti-GM2 antibodies. Overall, 88% of the patients developed IgM anti-GM2 antibodies; 71% also developed IgG anti-GM2 antibodies. GM2-KLH doses of 3–70 μg seemed to be equivalent in terms of peak titers and induction of anti-GM2 antibodies. At the 30-μg dose level, 50% of the patients developed complement fixing anti-GM2 antibodies detectable at a serum dilution of 1:10. We conclude that the GM2-KLH/QS21 formulation is more immunogenic than our previous formulation and that 3 μg is the lowest dose that induces consistent, high-titer IgM and IgG antibodies against GM2.

INTRODUCTION

GM2 is a ganglioside expressed on the surface of most melanomas and has been demonstrated to be immunogenic (1, 2). In our previous studies, we demonstrated that melanoma patients who were free of disease after complete surgical resection and who have natural or vaccine-induced antibodies to GM2 have a decreased risk of relapse (3). Immunization with GM2 alone does not induce antibodies (4); induction of optimal immunity against GM2 requires immunization with a potent adjuvant (5). In previous trials, GM2 was mixed with *bacille Calmette-Guérin*, which resulted in short-lived IgM antibodies (titers ≥ 1:80) in approximately 74% of patients, but rarely induced IgG antibodies against GM2 (approximately 10% of patients immunized; Ref. 3). Although IgM antibodies are potent mediators of CMC,[4] we hypothesized that the additional induction of an IgG response against GM2 could result in a more pronounced clinical effect. However, induction of IgG antibodies against carbohydrate antigens such as gangliosides would require a T_H epitope to provide the appropriate signals for immunoglobulin class switching.

To address this issue, GM2 was conjugated to KLH, a carrier protein known to provide T-cell help and administered with adjuvant QS21, a saponin fraction extracted from the bark of the South American tree *Quillaja saponaria Molina* (6). In two pilot studies using GM2 doses of 70 μg, this formulation resulted in high-titer IgG antibodies against GM2 (5, 7). Both IgM and IgG antibodies reacted with $GM2^+$ tumor cells by flow cytometry and induced complement-mediated lysis (8). In these two trials, 32 (76%) of 42 patients developed IgG antibodies against GM2 at titers ≥1:80 when doses of QS21 ≥100 μg were used. Thus, IgG antibodies could consistently be induced against GM2.

The objective of the current trial was to determine the minimal dose of GM2-KLH required for a consistent, high-titer IgM and IgG antibody response. This is one of the first dose-response studies carried out in patients receiving a defined cancer vaccine and identifies a dose that is appropriate for future Phase III trials.

Received 12/16/98; revised 8/23/99; accepted 12/13/99.
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[1] Supported by National Cancer Institute Grant PO1 CA33049.
[2] To whom requests for reprints should be addressed, at Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021. Phone: (212) 639-5015; Fax: (212) 794-4352.
[3] P. O. L. is a paid consultant and a shareholder in Progenics Pharmaceuticals.

[4] The abbreviations used are: CMC, complement-mediated cytotoxicity; KLH, keyhole limpet hemocyanin; AUC, area under curve, LDH; lactate dehydrogenase.

Table 1 Dose levels and formulations of GM2-KLH + QS21 vaccine

Dose level (μg of GM2)	No. of patients immunized
1	5
3	10
10	10
30	20[a]
70	7
Total	52

[a] The second 10 patients at the 30-μg dose level received vaccine in which the GM2-KLH and QS21 were vialed separately and mixed just prior to administration.

Table 2 Patient characteristics of 52 patients treated

Stage	
II (>4 mm)	4
III	39
IV	9
Gender	
Male/Female	34/18
Primary site	
Trunk	24
Extremity	20
Head/neck	6
Unknown	2
Median age (range)	60 (26–77)
Median time in months from complete resection until first vaccine (range)	5.7 (2.1–12)

MATERIALS AND METHODS

Vaccine Preparation

GM2-KLH was prepared with GM2 from bovine brain and supplied by Progenics Pharmaceuticals, Inc. (Tarrytown, New York) as described previously (5, 9). QS21 was supplied by Aquila BioPharmaceuticals (Framingham, MA).

In general, the vaccine was formulated in a single vial containing both GM2-KLH and QS21. However, a group of 10 patients immunized at the 30-μg dose level were immunized with GM2-KLH and QS21 vialed separately. For these patients, the GM2-KLH and QS21 were mixed by the pharmacist just prior to administration.

Patient Eligibility

Melanoma patients with American Joint Committee on Cancer stage III or IV, or deep stage II (>4 mm), who were free of disease after complete surgical resection were eligible. All of the pathology was confirmed by the Memorial Hospital Pathology Department. In general, patients were started on vaccine within 10 months of surgical resection, but patients were still eligible even after 10 months if their risk of relapse was felt to be >50%. All of the patients signed written informed consent.

Patients were excluded if their Karnofsky performance status was <80, if they had received systemic therapy or radiotherapy within the previous 8 weeks, or if they had a medical condition that would make it difficult to complete the full course of vaccination or to respond immunologically to the vaccine. Women who were pregnant or breast-feeding were not eligible.

Treatment Plan

This trial was carried out under an IND from the United States Food and Drug Administration. Within 4 weeks of starting vaccinations, patients had a physical exam, chest X-ray or chest CT, complete blood count, and comprehensive chemistry screen. An electrocardiogram was required within 10 months of starting the study.

Vaccines were administered by the Clinical Immunology nurses (Clinical Immunology Service, Memorial Sloan-Kettering Cancer Center) as a s.c. injection (final volume, 0.75 ml). Vaccinations were administered on weeks 1, 2, 3, 4, 12, 24, and 36.

This study was designed to compare the immunological effects of different doses of GM2-KLH vaccine. Groups of 5–10 patients were accrued to each of five vaccine dose levels in which the GM2-KLH concentration was adjusted to deliver a GM2 dose of 1, 3, 10, 30, or 70 μg (Table 1). All of the vaccinations contained 100 μg of QS21. Subsequently, the vaccine formulation was changed so that the GM2-KLH and QS21 were prepared in separate vials and mixed just prior to vaccine administration. Using this "two-vial system," an additional 10 patients were immunized at the 30-μg dose level.

Treatment Evaluation

Serological Analysis. Serum was collected immediately prior to each vaccination (including pretreatment), and on weeks 6, 13, 18, 26, 30, 38, and 42. In addition, serum was collected 3 and 6 months after the 7th and final vaccination. Anti-GM2 antibodies were measured using an ELISA method in which GM2 ganglioside is adsorbed to 96-well polystyrene microtiter plates. The remaining binding sites on the plate were blocked by PBS/casein/Tween 20 buffer. Serially diluted patient sera or controls were added, and bound antibody was detected using a goat antihuman IgM or IgG antibody (heavy-chain-specific) conjugated to alkaline phosphatase. Plates were developed using *p*-nitrophenyl phenol substrate, and absorbance was read at 405 nm with a correction of 620 nm. Antibody titer was defined as the highest dilution of patient serum yielding a corrected absorbance of 0.1. Pooled human serum from previously vaccinated patients with a known anti-GM2 antibody titer or pooled normal human serum with no anti-GM2 reactivity were used as positive and negative controls, respectively. A positive serological response was defined as an anti-GM2 titer ≥ 1:80 observed at two or more time points.

The antibody titers plotted *versus* time were also analyzed as the AUC using Prism version 2.01 software (Graph Pad Software, Inc., San Diego, CA). The AUC of the antibody response was considered to represent the overall exposure to anti-GM2 IgG or IgM over time.

CMC Assay. CMC assays were performed by the LDH release method (Boehringer-Mannheim). SK-MEL31 (GM2-positive) or SK-MEL24 (GM2-negative) were plated in 96-well tissue culture plates and incubated at 37°C in a humidified CO_2 incubator. The medium was removed, and plain DMEM containing human serum complement standard (Sigma Chemical Co., St. Louis, MO) was added along with the pre- or postim-

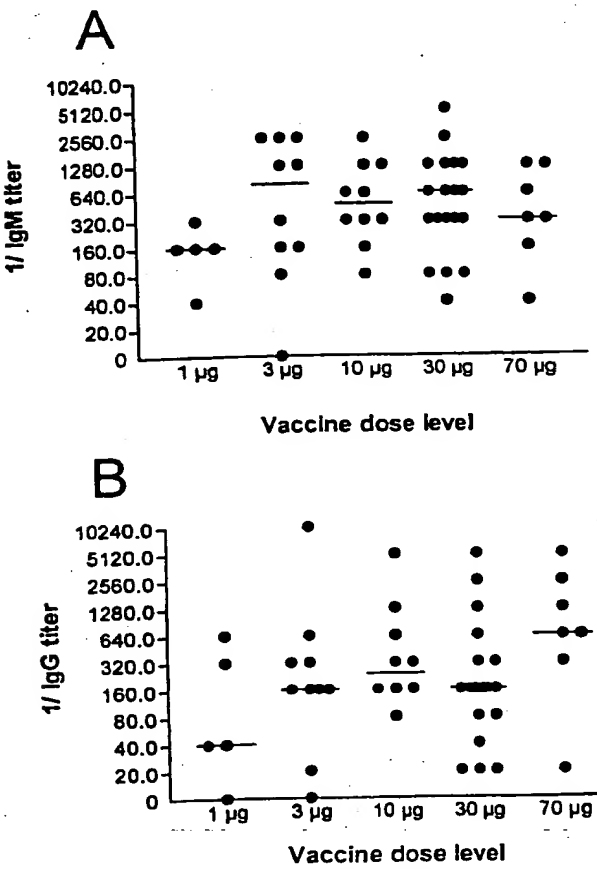

Fig. 1 Peak anti-GM2 antibody titers in patients immunized with GM2-KLH + QS21 at GM2 doses of 1, 3, 10, 30, or 70 µg. *Each dot*, a single patient. The *horizontal lines*, the median peak titers for each dose level. *A*, peak IgM titers; *B*, peak IgG titers.

munization serum to be tested in duplicate wells. The postimmunization serum tested was the serum sample showing the highest IgM anti-GM2 titers for each patient. Both the complement and serum were used at a final dilution of 1:10. In positive control wells, 1% NP40 was added to measure maximal release. The plate was returned to the incubator for 16 h. The supernatants were removed and transferred to a 96-well ELISA plate for analysis. LDH substrate/catalyst was added, and the plate was incubated in the dark at 25°C for 20 min. The plate was read on a spectrophotometer at 492 nm. Each patient's preimmune CMC reading served as the control for the postimmune CMC result. Percent-specific lysis against each cell line was calculated as follows:

$$\frac{\text{(Postimmune serum LDH release} - \text{preimmune serum LDH release)}}{\text{NP40 LDH release}}$$

Clinical Evaluation. Patients were evaluated clinically at Memorial Hospital on weeks 12, 24, and 36 and on three months after the 7th vaccination. A chest X-ray, complete blood count, and comprehensive screening profile were repeated at the time of the 5th and 7th vaccination; an electrocardiogram was repeated at the time of the 7th vaccination. Toxicity was scored using standard criteria (10).

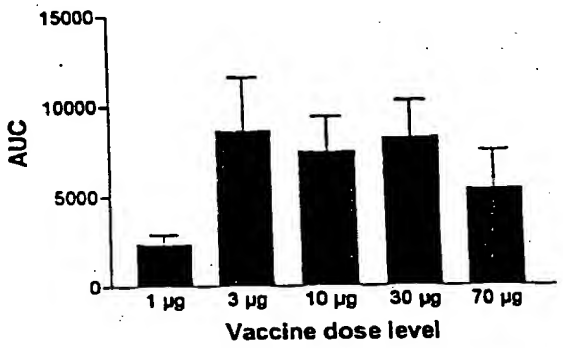

Fig. 2 AUC analysis for the IgM anti-GM2 responses at each dose level. The AUC was calculated for each patient up to week 30. *Height of the columns*, the mean (± SE) AUC for each dose level.

RESULTS

Patient Characteristics. Fifty-two patients were entered on this trial between January 1995 and April 1996 (Table 2). There were 34 men and 18 women. Most (75%) of the patients had stage III melanoma; 8% had deep stage II, and 17% had stage IV. The patients had been free of disease for a median of 5.7 months before beginning the trial.

Serological Results. Applying rigorous definitions of response (defined in "Materials and Methods") 88% of the patients immunized in this study developed an IgM response against GM2; 71% developed an IgG response. Fig. 1 shows the peak anti-GM2 titers attained at each dose level. For IgM, the median peak titers ranged from 1:160 to 1:800; for IgG the median peak titers ranged from 1:40 to 1:640. When comparing the incidence of nonresponding patients (peak titers ≤1:40) for IgM and IgG at each of the dose levels, we found no difference for the IgM response ($P = 0.73$; χ^2) or IgG response ($P = 0.19$; χ^2). From the exploratory analysis, it appeared that there were fewer IgG responses at the 1-µg dose level.

An AUC analysis was performed for both IgM and IgG anti-GM2 responses on each patient until week 30, and the mean AUCs at each dose level were compared. For the IgM anti-GM2 response, the mean AUC at the 1-µg dose level was lower than the mean AUC at any of the other dose levels (Fig. 2). The mean AUC for the IgG response was also lower in patients treated at the 1-µg dose level compared with the mean AUCs at the other dose levels (data not shown), but this difference was not statistically significant. There were no differences in the AUC for the other dose levels.

Given that the 1-µg dose level seemed to have a lower incidence of inducing IgG against GM2 and a lower mean AUC for the IgM response, we concluded that the 1-µg dose level was less immunogenic than the other dose levels. As a result, we focused on the 3-, 10-, 30-, and 70-µg dose levels.

Fig. 3 illustrates the median anti-GM2 IgM and IgG titers for patients immunized at the 3-, 10-, 30-, and 70-µg dose

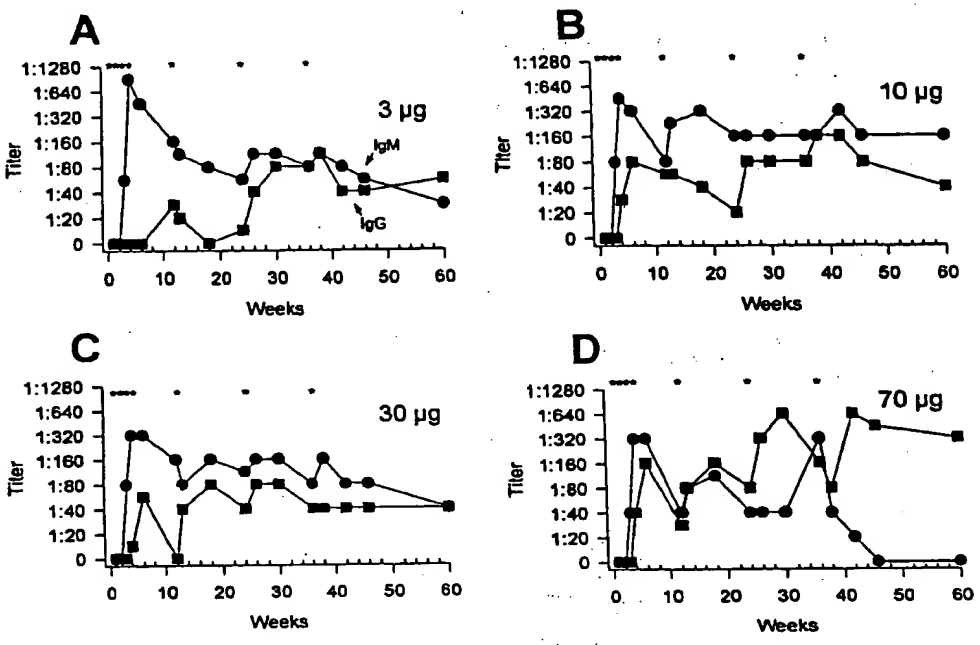

Fig. 3 Median anti-GM2 antibody titers in patients immunized with GM2-KLH + QS21 at GM2 doses of 3 μg (*A*), 10 μg (*B*), 30 μg (*C*), or 70 μg (*D*). IgM titers (●) and IgG titers (■) are shown separately at each dose level. *, administration of vaccine.

levels. At these four dose levels, there was a consistent IgM response followed by an IgG response. Both the IgM and IgG responses were sustained for months after the final immunization. At week 60 (5½ months after the last immunization), serum was available on 20 patients who had developed an IgM response and 19 patients who had developed an IgG response. Analysis of these sera showed that the IgM response persisted in 45% of the cases; the IgG response persisted in 53% of the cases (data not shown). This demonstrates that, in one-half of the patients who developed anti-GM2 antibodies, the antibody response persisted for at least 5½ months.

Most of the patients immunized on this trial received vaccine that had been formulated in one vial (*i.e.*, GM2-KLH and QS21 were stored together). However, 10 of the 20 patients immunized at the 30-μg dose level received vaccine formulated in two vials because we obtained evidence that the stability of the vaccine was enhanced if the GM2-KLH and QS21 were stored in separate vials and mixed just prior to vaccine administration. We compared the anti-GM2 response induced in patients immunized with the single-vial *versus* the two-vial formulation at the 30-μg dose level (Fig. 4). The median IgM titers were similar in the two groups; the median IgG titers were slightly lower in the group receiving vaccine formulated as two vials. All of the patients immunized with the single-vial formulation developed anti-GM2 antibodies, and only one patient immunized with the two-vial formulation failed to develop anti-GM2 antibodies. We conclude that there was no difference in the immunogenicity between the one-vial and the two-vial formulations.

CMC. Sera from 18 of the 20 patients treated at the 30-μg dose level were available to be tested for the ability to bind melanoma cells and to fix the complement (Fig. 5). In 9 of the 18 patients, the postvaccination sera showed an increase in

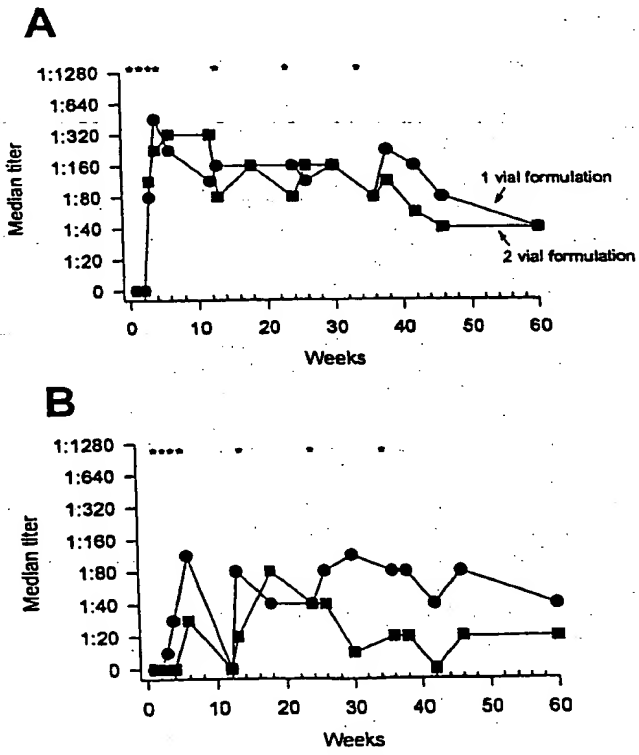

Fig. 4 Comparison of median anti-GM2 IgM titers (*A*) and IgG titers (*B*) among patients immunized at the 30-μg dose level. , patients immunized with vaccine formulated in a single vial; ■, patients immunized with vaccine formulated in 2 vials, in which the GM2-KLH and QS21 vialed separately; *, administration of vaccine.

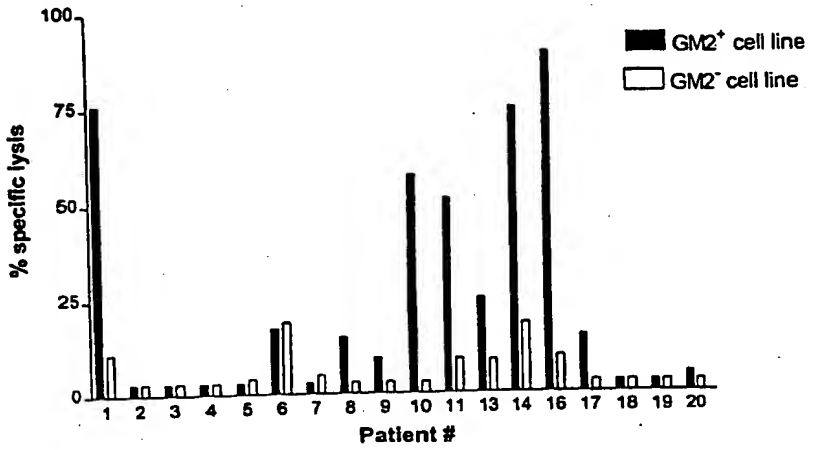

Fig. 5 CMC of sera from patients immunized at the 30-μg dose level. ■, the increase of CMC against a $GM2^+$ cell target in postvaccination sera compared with pretreatment sera. □, the increase of CMC against a $GM2^-$ cell target in postvaccination sera compared with pretreatment sera. Data for patients 12 and 15 are not available.

CMC compared to pretreatment that was specific for the $GM2^+$ cell target. In the remaining nine patients, there was either no increase in CMC compared with pretreatment levels (patients 2, 3, 4, 5, 7, 18, 19, and 20) or the increase was not specific for GM2 (patient 6). Induction of complement-fixing activity correlated with a peak IgM anti-GM2 titer of 1:640. All of the nine patients demonstrating CMC activity in their serum had peak IgM anti-GM2 titers ≥ 1:640 as opposed to only two of nine patients without CMC activity ($P = 0.002$; Fisher's exact test).

Toxicity. Virtually all of the patients experienced inflammation and/or pruritis at the site of injection attributed to the known effects of the QS21 adjuvant (7). Other common side effects were: (*a*) fever (71%); (*b*) mild fatigue (44%) and flu-like symptoms (58%); (*c*) chills (29%); and (*d*) myalgias (48%). These were self-limiting, never more severe than grade 2, and rarely lasted more than 24 h. Headache was seen in 66% of the patients and was grade 1–2 except in one patient with a grade-3 headache. These toxicities were felt to be due largely to QS21, which is consistent with the observation that there was no correlation between vaccine dose and toxicity. Grade 3 or 4 toxicity possibly related to vaccine occurred in four patients. One patient developed transient dyspnea, which resolved spontaneously. Another patient reported 2–3 days of severe dizziness, which also resolved spontaneously. One patient developed atrial flutter while on the study and required treatment. A fourth patient, with a history of migraine headaches, reported a grade 3 headache associated with vaccine therapy. No patient was taken off study because of toxicity.

DISCUSSION

The current trial confirms that vaccinating melanoma patients with GM2-KLH + QS21 induces both IgM and IgG antibodies against GM2. We observed that 88% of patients developed IgM anti-GM2 antibodies and 71% developed IgG anti-GM2 antibodies. This compares almost exactly with the immunological results observed in our previous pilot trials (5, 7). Because the previous trials used vaccine produced at Memorial Sloan-Kettering Cancer Center and the current trial used vaccine produced by Progenics Pharmaceuticals, Inc., this demonstrates that subsequent lots of the vaccine can be produced successfully and that the immunogenicity is reproducible. The results also show that the vaccine can be formulated either with QS21 or vialed separately and mixed with QS21 just prior to administration. We favor formulating GM2-KLH and QS21 in separate vials because of improved stability.

This is one of the first cancer vaccine trials to explore dose-response effects using a defined antigen. Our previous trials used GM2-KLH at a GM2 dose of 70 μg and demonstrated that all of the patients developed IgM anti-GM2 and 76% developed IgG anti-GM2. In this current trial, we have explored GM2 doses of 1, 3, 10, 30, and 70 μg. We conclude that the immunogenicity of GM2-KLH at a GM2 dose of 1 μg is suboptimal based on the fact that the 1-μg dose was less likely to induce IgG anti-GM2 antibodies. The mean AUC for the anti-GM2 IgM antibody responses was also lowest for the 1-μg dose level, which implies that this dose resulted in the lowest level of tumor-cell exposure to anti-GM2 antibody. At the higher vaccine doses (3, 10, 30, or 70 μg), there was no apparent difference in the immunogenicity of the vaccine. Peak titers, AUC, antibody responses over 60 weeks, and percent of nonresponding patients were similar at the 3-, 10-, 30-, and 70-μg dose levels.

In patients immunized at the 30-μg dose level, 50% of the patients developed antibodies that fixed complement and resulted in CMC against $GM2^+$ melanoma. CMC activity correlated with peak IgM anti-GM2 titers ≥1:640. This demonstrates that immunization induced anti-GM2 antibodies capable of binding cell-surface GM2 and mediating effector functions.

In at least one-half of the patients, the anti-GM2 antibody response persisted for more than 5½ months. This is consistent with the notion that the KLH carrier protein provides sufficient T-cell help to induce a more prolonged antibody response against GM2. It is also important to note that patients at the 70-μg dose level received a 23-fold higher KLH dose compared with patients at the 3-μg dose level, and that this was not associated with any excessive toxicity or decreased immunogenicity. This is reassuring as we consider construction of multivalent vaccines containing 4 or 5 antigens conjugated to KLH. Our results suggest that these higher

total KLH doses will neither be more toxic nor lead to diminished immunogenicity.

These studies provide a basis for additional trials with GM2-KLH + QS21. Future clinical trials will examine the effects of IFN-α on the anti-GM2 response induced by GM2-KLH + QS21, the immunogenicity of GM2-KLH + QS21 combined with GD2-KLH, and a Phase III trial comparing GM2-KLH + QS21 to IFN-α for the ability to prevent recurrence of melanoma in stage III patients. For these trials, a vaccine dose ≥3 μg of GM2 should be used.

ACKNOWLEDGMENTS

We are grateful to the Immunology Nurses at Memorial Sloan-Kettering Cancer Center who dedicated much effort to the implementation of this protocol.

REFERENCES

1. Tai, T., Cahan, L. D., Tsuchida, T., Saxton, R. E., Irie, R. F., and Morton, D. L. Immunogenicity of melanoma-associated gangliosides in cancer patients. Int. J. Cancer, *35:* 607–612, 1985.

2. Livingston, P. O., Ritter, G., Oettgen, H. F., and Old, L. J. Immunization of melanoma patients with purified gangliosides. *In:* H. F. Oettgen (ed.), Gangliosides and Cancer, pp. 293–300. New York: VCH Publishers, Inc., 1989.

3. Livingston, P. O., Wong, G. Y. C., Adluri, S., Tao, Y., Padavan, M., Parente, R., Hanlon, C., Jones Calves, M., Helling, F., Ritter, G., Oettgen, H. F., and Old, L. J. Improved survival in stage III melanoma patients with GM2 antibodies: a randomized trial of adjuvant vaccination with GM2 ganglioside. J. Clin. Oncol., *12:* 1036–1044, 1994.

4. Livingston, P. O., Natoli, E. J., Jr., Calves, M. J., Stockert, E., Oettgen, H. F., and Old, L. J. Vaccines containing purified GM2 ganglioside elicit GM2 antibodies in melanoma patients. Proc. Natl. Acad. Sci. USA, *84:* 2911–2915, 1987.

5. Helling, F., Zhang, S., Shang, A., Adluri, S., Calves, M., Koganty, R., Longenecker, B. M., Yao, T-J., Oettgen, H. F., and Livingston, P. O. GM2-KLH conjugate vaccine: increased immunogenicity in melanoma patients after administration with immunological adjuvant QS-21. Cancer Res., *55:* 2783–2788, 1995.

6. Kensil, C. R., Patel, U., Lennick, M., and Marciani, D. Separation and characterization of saponins with adjuvant activity from *Quillaja saponaria Molina* cortex. J. Immunol., *146:* 431–437, 1991.

7. Livingston, P. O., Adluri, S., Helling, F., Yao, T. J., Kensil, C. R., Newman, M. J., and Marciani, D. Phase 1 trial of immunological adjuvant QS-21 with a GM2 ganglioside-keyhole limpet haemocyanin conjugate vaccine in patients with malignant melanoma. Vaccine, *12:* 1275–1280, 1994.

8. Livingston, P., Zhang, S., Adluri, S., Yao, T-J., Graeber, L., Ragupathi, G., Helling, F., and Fleisher, M. Tumor cell reactivity mediated by IgM antibodies *in sera* from melanoma patients vaccinated with GM2 ganglioside covalently linked to KLH is increased by IgG antibodies. Cancer Immunol. Immunother., *43:* 324–330, 1997.

9. Helling, F., Shang, A., Calves, M., Zhang, S., Ren, S., Yu, R. K., Oettgen, H. F., and Livingston, P. O. Increased immunogenicity of GD3 conjugate vaccines: comparison of various carrier proteins and selection of GD3-KLH for further testing. Cancer Res., *54:* 197–203, 1994.

10. Creekmore, S. P., Longo, D. L., and Urba, W. J. Principles of the clinical evaluation of biologic agents. *In:* V. T. J. DeVita, S. Hellman, and S. A. Rosenberg (eds.), Biologic Therapy of Cancer, pp. 67–86. Philadelphia: J. B. Lippincott Company, 1991.